Broad spectrum antibiotic enrofloxacin modulates contact sensitivity through gut microbiota in a murine model

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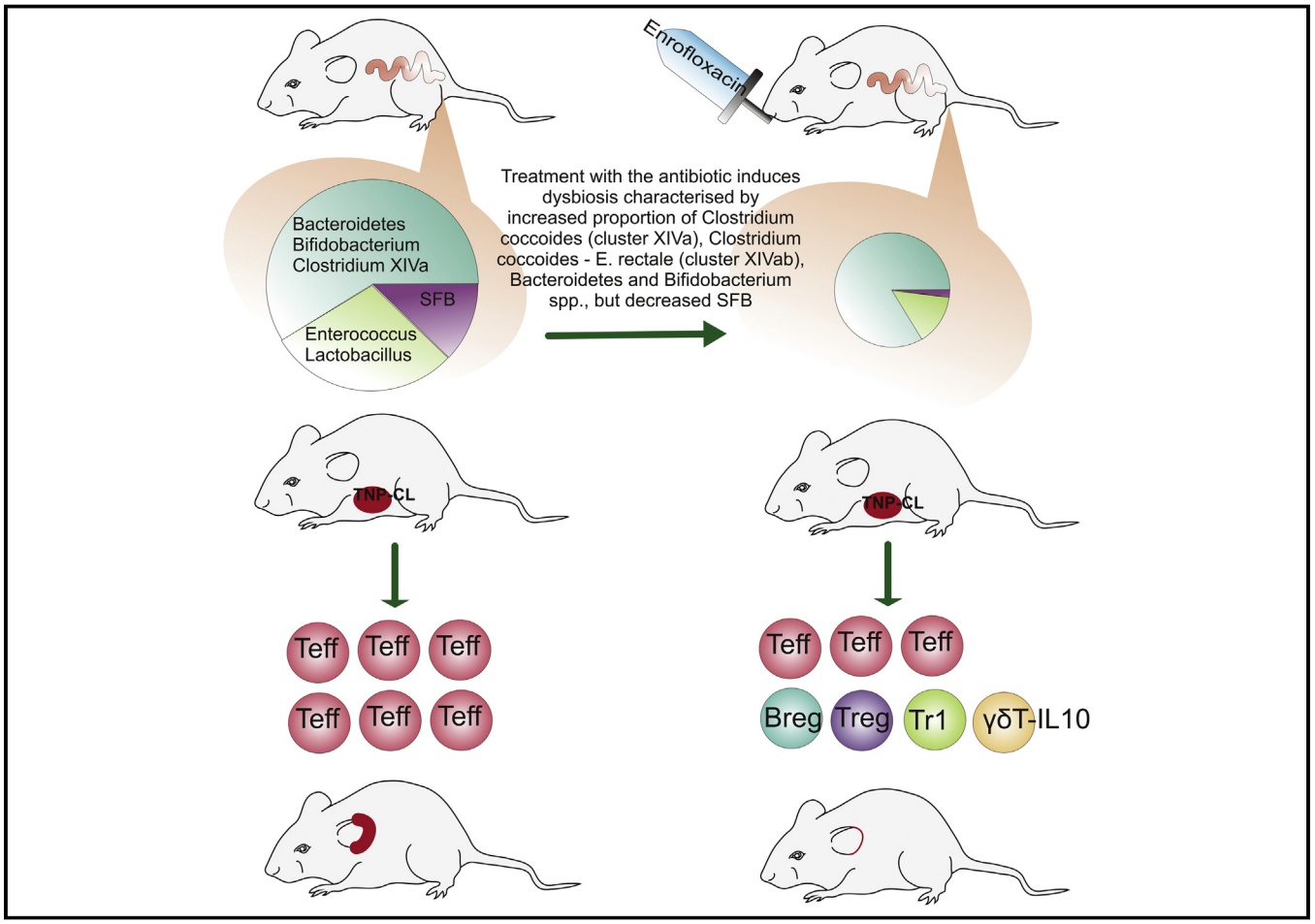
GRAPHICAL ABSTRACT



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Background: Medical advances in the field of infection therapy have led to an increasing use of antibiotics, which, apart from eliminating pathogens, also partially eliminate naturally existing commensal bacteria. It has become increasingly clear that less exposure to microbiota early in life may contribute to the observed rise in "immune-mediated" diseases, including autoimmunity and allergy.
Objective: We sought to test whether the change of gut microbiota with the broad spectrum antibiotic enrofloxacin will modulate contact sensitivity (CS) in mice.
Methods: Natural gut microbiota were modified by oral treatment with enrofloxacin prior to sensitization with trinitrophenyl chloride followed by CS testing. Finally, adoptive cell transfers were performed to characterize the regulatory cells that are induced by microbiota modification.
Results: Oral treatment with enrofloxacin suppresses CS and production of anti–trinitrophenyl chloride IgG1 antibodies. Adoptive transfer experiments show that antibiotic administration favors induction of regulatory cells that suppress CS. Flow cytometry and adoptive transfer of purified cells show that antibiotic-induced suppression of CS is mediated by TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg, $CD19^+B220^+CD5^+$ IL-10^+, IL-10^+ Tr1, and IL-10^+ TCR $\gamma\delta^+$ cells. Treatment with the antibiotic induces dysbiosis characterized by increased proportion of *Clostridium coccoides* (cluster XIVa), *C coccoides–Eubacterium rectale* (cluster XIVab), *Bacteroidetes,* and *Bifidobacterium* spp, but decreased segmented filamentous bacteria. Transfer of antibiotic-modified gut microbiota inhibits CS, but this response can be restored through oral transfer of control gut bacteria to antibiotic-treated animals.
Conclusions: Oral treatment with a broad spectrum antibiotic modifies gut microbiota composition and promotes anti-inflammatory response, suggesting that manipulation of gut microbiota can be a powerful tool to modulate the course of CS. (J Allergy Clin Immunol 2017;140:121-33.)

Key words: *Contact sensitivity, gut microbiota, immunoregulation, dysbiosis, regulatory cells*

Abbreviations used
AD: Atopic dermatitis
ALNC: Axillary and inguinal lymph node cell
Breg: Regulatory B
CS: Contact sensitivity
EAE: Experimental autoimmune encephalomyelitis
FACS: Fluorescence-activated cell sorting
i.p.: Intraperitoneal
i.v.: Intravenous
MLNC: Mesenteric lymph node cell
MPO: Myeloperoxidase
PP: Peyer patch
SFB: Segmented filamentous bacteria
SPLC: Splenocyte
T_H: T helper lymphocyte
Treg: Regulatory T
TNP-Cl: Trinitrophenyl chloride

Advances in medicine in the field of infection therapy over the last several decades have led to a drastically increasing application of antibiotics, which, apart from eliminating pathogens, also partially eliminate naturally existing commensal bacteria. In addition, unlabeled broad-spectrum antibiotics are often present in the food supply, which also can change the composition of gut microbiota. A study in a group of healthy human volunteers showed that treatment with ciprofloxacin for 5 days decreases diversity, richness, and evenness of fecal microbiota during antibiotic treatment.[1] Although the microbiota mostly returned to pretreatment composition following ciprofloxacin treatment, several bacterial taxa failed to recover, indicating that changes to the microbiota can persist following a short course of oral antibiotics.[2] Similar to human studies, mouse models have revealed that treatment with ampicillin, cefoperazone, vancomycin, or clindamycin causes long-lasting changes in the gut microbiota that persist after cessation of antibiotic treatment.[3-5]

It has become increasingly clear that less exposure to microbiota early in life may contribute to the observed rise in the diseases characterized by immune dysregulation, including allergy, autoimmunity, metabolic disorders, and even neoplastic diseases.[6] A number of studies have demonstrated the increased risk of developing asthma and food allergy in children who have been exposed to antibiotics in the first year of life.[7-9] Similar findings are also observed in animal studies, such as in the non-obese-diabetic mouse, which is known to be very sensitive to the change of the environment, such that a cleaner environment enhances the rate of onset and incidence of type 1 diabetes.[10] This is also the case in animal models of allergic asthma.[11,12] Studies carried out in recent years have confirmed a strong connection between the natural gut microbiota and the immune response. However, this interaction may differ in various experimental models. In an animal model of multiple sclerosis, for instance, a strong inhibition of inflammation has been observed in mice treated orally with an antibiotic.[13] Similar observations have been reported in an animal model of human rheumatoid arthritis.[14]

Atopic dermatitis (AD) is a prominent clinical example of an immune-mediated disease that is affected by environmental factors. AD has become a considerable clinical problem, having nearly tripled in prevalence over the last 30 years in developed countries. The disease afflicts 15% to 30% of children and about 2% to 10% of adults.[15] Yet another form of skin disease with an underlying immune-mediated hypersensitivity reaction is contact sensitivity (CS) to haptens. Allergic contact dermatitis, as opposed to AD, is a typical CS response in humans and has become a significant cause of morbidity. Indeed, contact dermatitis linked to exposure to chemical substances in the workplace constitutes about 30% of all occupational diseases and represents a severe social and economic problem.[16]

The hygiene hypothesis has been advanced as an explanation for the dramatic rise in the prevalence of allergic diseases in industrialized societies. Supporters of this hypothesis note that decreasing microbial exposure of many types, including bacteria, parasites, and viruses, has been associated with the rise in incidence of allergic diseases and autoimmune disorders.[17] Increasing evidence suggests that gut microbes, which represent an enormous source of interaction between microbes and immunity, play a very important role in various health problems.[6] However, it is not clear what effects are exerted by natural gut microbiota on the course of CS responses. Given the common use of antibiotics in industrialized societies, in the setting of ever-increasing exposure to potential CS-generating haptens in

the "chemicalized" modern environment, we see that it is important to study the effect of the gut microbiota on the CS response. In the current article, therefore, we test our hypothesis that change of gut microbiota with a broad spectrum antibiotic will modulate CS response in mice.

METHODS

Mice

C57BL/6 mice, at 6 weeks old, were taken from the breeding unit of the Department of Medical Biology, Jagiellonian University, College of Medicine. Mice were kept under pathogen-free conditions in individual cages using Aero-Mouse IVC Green Line system (Tecniplast SpA, Buguggiate, Italy). All experiments were conducted in compliance with the guidelines of the First Local Ethics Committee of the Jagiellonian University Medical College.

Reagents

Trinitrophenyl chloride (TNP-Cl) (Chemica Alta, Edmonton, Alberta, Canada); Evans blue, formamide, hexadecyltrimethylammonium bromide, lysozyme, magnesium chloride, JumpStart Taq ReadyMix for quantitative PCR, o-dianisidine dihydrochloride, sodium dodecyl sulfate, Tris-EDTA, and hydrogen peroxide were obtained from Sigma (St Louis, Mo). Silica beads were from BioSpec Products (Bartlesville, Okla); proteinase K was from Roche Diagnostics (Mannheim, Germany). Baytril (enrofloxacin) was purchased from Bayer Animal Healthcare GmbH (Leverkusen, Germany).

Sensitization and elicitation of CS *in vivo*

Contact sensitivity to TNP-Cl was induced as described previously.[18] Vascular permeability, myeloperoxidase (MPO) assay, and concentration of IFN-γ was measured as previously described.[18]

Treatment with enrofloxacin

Mice received drinking water containing the broad spectrum antibiotic enrofloxacin (0.27 mg/mL) or water alone for 2 weeks prior to sensitization with TNP-Cl. In some experiments, mice received enrofloxacin intraperitoneally ([*i.p.*]; 5 mg/kg) or PBS for 2 weeks prior to further tests.

Evaluation of gut flora depletion

Serial dilutions of colon contents from enrofloxacin-treated or untreated mice were cultured on general agar plates (blood agar, Bioshop Inc, Burlington, Ontario, Canada) at 37°C for 48 hours or 72 hours in aerobic and anaerobic conditions, respectively. Total bacteria per gram of sample was calculated using colony forming unit.

Adoptive cell transfer of CS and cell mixing assay to evaluate suppression (transfer out protocol). Donors of CS-immune effector cells were contact sensitized with 5% TNP-Cl. Axillary and inguinal lymph nodes (ALNs) were harvested from TNP-Cl-immune mice on day +4, and 7×10^7 immune cells were incubated for 30 minutes at 37°C in RPMI 1640 medium alone, washed, and then injected *i.v.* into normal syngeneic recipients (positive transfer). For the cell mixing assay, 7×10^7 of the CS-effector immune cells from TNP-Cl contact sensitized donors were incubated for 30 minutes at 37°C with 5×10^7 ALNCs or splenocytes (SPLCs), or 1×10^7 Peyer patch (PP) cells or mesenteric lymph node cells (MLNCs), from mice treated orally with enrofloxacin for 2 weeks prior to TNP-Cl immunization. After incubation, the cell mixture was transferred *i.v.* into naive recipients. Mice were challenged with the hapten within 30 minutes after cell transfer and tested for CS 24 hours later.

Transfer of regulatory cells prior to CS induction or elicitation to confirm active tolerance (transfer in protocol). To confirm data found in the "transfer out" cell mixing assay, 5×10^7 ALNCs or SPLCs, or 1×10^7 PP cells or MLNCs, isolated from donors orally treated with enrofloxacin for 2 weeks prior to TNP-Cl sensitization, were transferred *i.v.* into syngeneic recipients 2 hours prior to CS induction, or 1 day prior to CS elicitation (day +3). Mice then underwent allergen challenge on the ears and were tested for CS. In some experiments, cell populations purified by magnetic cell sorting were transferred as indicated to characterize their regulatory activity.

Extraction of bacterial DNA from the gut content

Gut content was collected by lavage with sterile PBS and centrifuged. The pellet was resuspended in sterile PBS and mixed vigorously. After centrifugation, the supernatant without the solid fraction was collected and centrifuged. The pellet was resuspended in 300 μL of Tris-EDTA. Samples were frozen and thawed several times followed by chemical disruption with lysozyme and SDS, followed by protein removal using silica beads. DNA extraction with phenol-chloroform-isoamyl alcohol (25:24:1) solution was performed as described elsewhere.

PCR conditions

To evaluate dysbiosis after oral antibiotic administration, RT-PCR was performed with 10.5 ng of DNA using CFX96 Touch (Bio-Rad Laboratories, Inc, Hercules, Calif). Detection of selected gut bacteria species and group was based on amplification of conserved 16S ribosomal DNA fragments. The primer sequences are summarized in Table E1 found in this article's Online Repository at www.jacionline.org. PCR conditions were as described previously.[19,20] The 16S ribosomal DNA detected by universal primers and probes, detecting all bacteria, was used as the reference gene.[21] The expression level was determined by the values received from mixed DNA samples extracted from control mice and presented as ΔΔct.[22] Values for all bacteria were subtracted from values obtained for selected bacteria.[23]

Transfer of gut microbiota

Cohousing was performed by housing mice given oral enrofloxacin with untreated animals for 2 weeks prior to TNP-Cl sensitization and CS test. The experiment of "adoptive microbiota transfer" (fecal microbiota transplantation) was performed by oral gavage with the fecal material from the donors that were or were not treated *per os* with enrofloxacin. Twenty-four hours after stopping enrofloxacin, the lumen contents of the large intestine were harvested in 4 mL of sterile PBS, mixed vigorously, and centrifuged. The fecal supernatant (300 μL) was orally inoculated twice a week for 2 weeks[24] prior to CS induction.

Statistics

Data in graphs are shown as mean ± SE. Student *t* test or ANOVA followed by Tukey multiple comparison test was used. Statistical significance was set at $P < .05$.

Description of all of the materials and methods used in this study can be found in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Oral application of broad spectrum antibiotic enrofloxacin alleviates CS in mice

To determine the influence of gut microbiota on CS, mice were treated orally or *i.p.* with the broad spectrum antibiotic enrofloxacin prior to TNP-Cl sensitization. Oral treatment with enrofloxacin reduced CS, as measured by ear swelling, in actively immunized mice (Fig 1 *A*, Group D vs B), whereas *i.p.* injection of enrofloxacin does not affect ear swelling (Group F vs B). Both oral or *i.p.* enrofloxacin treatment did not affect the body weight of mice, regardless of TNP-Cl immunized, when compared with animals not treated with enrofloxacin (Fig 1, *B*, Groups C and E vs A and Groups D and F vs B). Oral antibiotic application

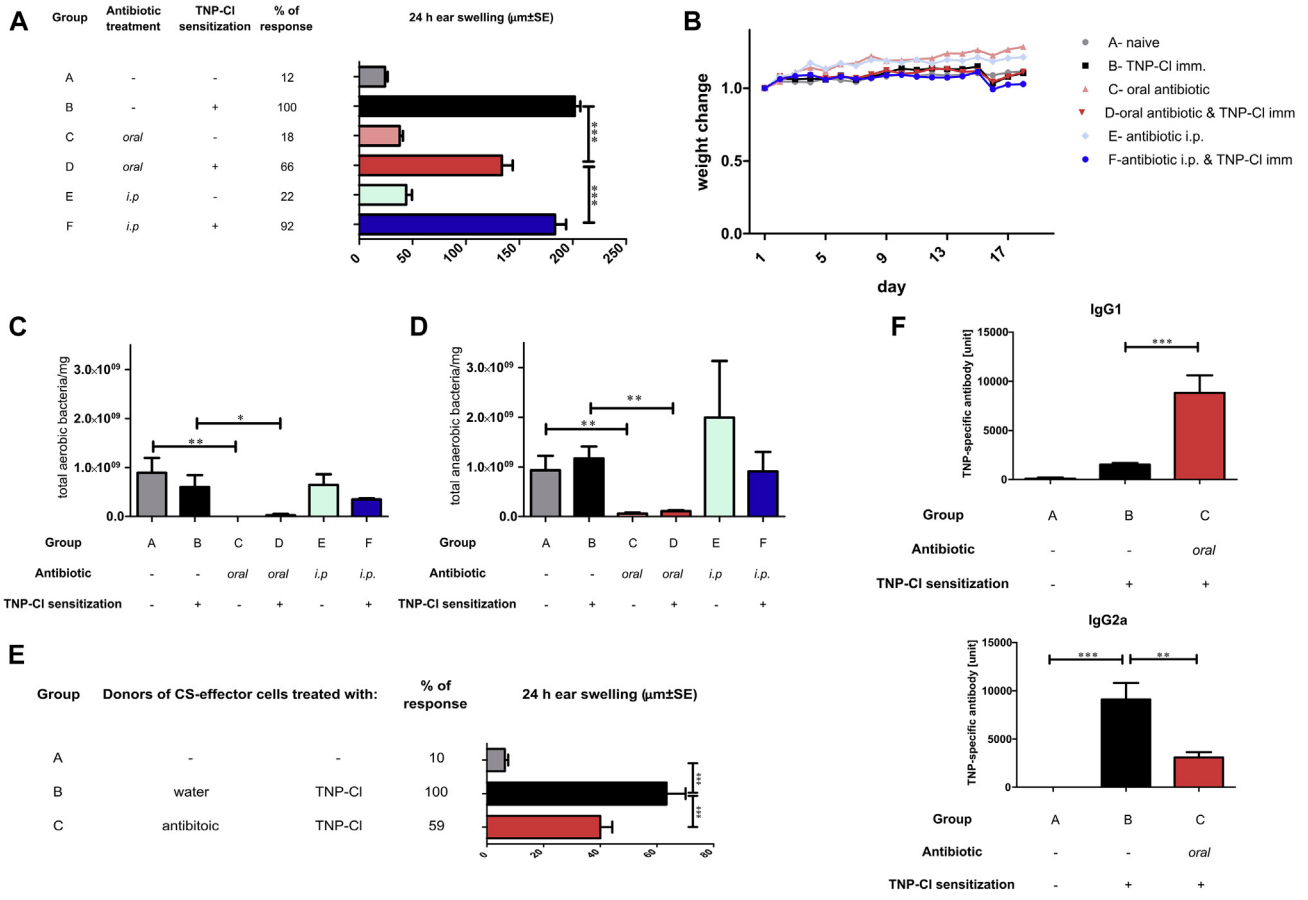


FIG 1. Oral application of broad spectrum antibiotic alleviates CS in mice. Mice received plain water (Groups A and B) or water containing enrofloxacin (0.27 mg/mL) (Groups C and D), or they were *i.p.* injected with enrofloxacin (5 mg/kg) (Groups E and F) for 2 weeks prior to TNP-Cl or sham immunization and test. **A,** Ear swelling. **B,** Body weight. **C** and **D,** Aerobic and anaerobic bacteria in content of large intestine. **E,** Adoptive transfer of CS. **F,** Production of TNP-specific IgG1 and IgG2a. Results shown as mean ± SE. n = 8 to 16 **(A)**, n = 5 to 6 **(B-D)**, n = 14 **(E)**, and n = 5 to 7 **(F)**. **P* < .05, ***P* < .01, and ****P* < .001.

significantly reduced the number of both aerobic (Fig 1, *C*) and anaerobic (Fig 1, *D*) bacteria in colon contents (Groups C and D vs A and B). Intraperitoneal injection of antibiotic did not affect bacterial counts of aerobes (Fig 1, *C*) and anaerobes (Fig 1, *D*, Groups E and F vs A and B). A decrease of CS in actively immunized mice due to antibiotic treatment was confirmed by adoptive lymphocyte transfer experiments. Transfer of axillary and inguinal lymph node cells (ALNCs) and SPLCs isolated from TNP-Cl sensitized donors that were orally treated with enrofloxacin to naive mice significantly reduced CS when compared with positive control (ALNC and SPLC from hapten-sensitized mice not treated with antibiotic) (Fig 1, *E*, Group C vs B). Additionally, oral treatment with antibiotic prior to CS induction and elicitation increased production of TNP-specific IgG1 antibodies but reduced synthesis of anti-TNP IgG2a antibodies (Fig 1, *F*, Group C vs B). The ear swelling data were confirmed in further experiments showing that oral treatment with enrofloxacin prior to TNP-Cl sensitization results in decreased vascular permeability (Fig 2, *A*) and lower ear weight (Fig 2, *B*). Additionally, oral application of enrofloxacin before induction of CS resulted in reduced MPO activity, as well as decreased IFN-γ concentration in ear extracts (Fig 2, *C* and *D*).

Oral treatment with antibiotic induces cells that inhibit CS *in vivo*. To test whether decreased CS responses after antibiotic treatment can be transferred into naive recipients, we performed adoptive transfer ("transfer out") experiments, in which naive recipient mice were given TNP-Cl immune cells (7×10^7 cells) that were preincubated with ALNCs or SPLCs (5×10^7), or MLNCs (1×10^7 cells), or PP-derived lymphocytes (1×10^7 cells) isolated from syngeneic animals that were orally treated with antibiotic and sensitized with TNP-Cl (Fig 3, *A*, Groups C-F). The recipient mice were tested for CS immune response after cell transfer. Mice in the positive control group received immune cells only (Group B). ALNCs, SPLCs, MLNCs, and PP cells from donors treated with antibiotic prior to TNP-Cl immunization inhibit the effector function of TNP-Cl-sensitized CS-effector cells (Fig 3, *A*, Groups C-F vs B). Additionally, employing a "transfer in" protocol, we showed that injection of ALNCs, SPLCs, MLNCs, or PP cells isolated from donors treated with antibiotic and sensitized with TNP-Cl into actively sensitized recipients 1 day before allergen challenge to the ear (day +3) suppressed CS response (Fig 3, *B*, Groups C-F vs B). Our results suggest that cells from antibiotic-treated mice prior to TNP-Cl sensitization suppress the effector phase of the CS response. To determine whether the antibiotic-induced cells also play a role during the CS induction phase, ALNCs, SPLCs, MLNCs, and PP cells isolated from mice receiving antibiotic prior to TNP-Cl sensitization were transferred into syngeneic

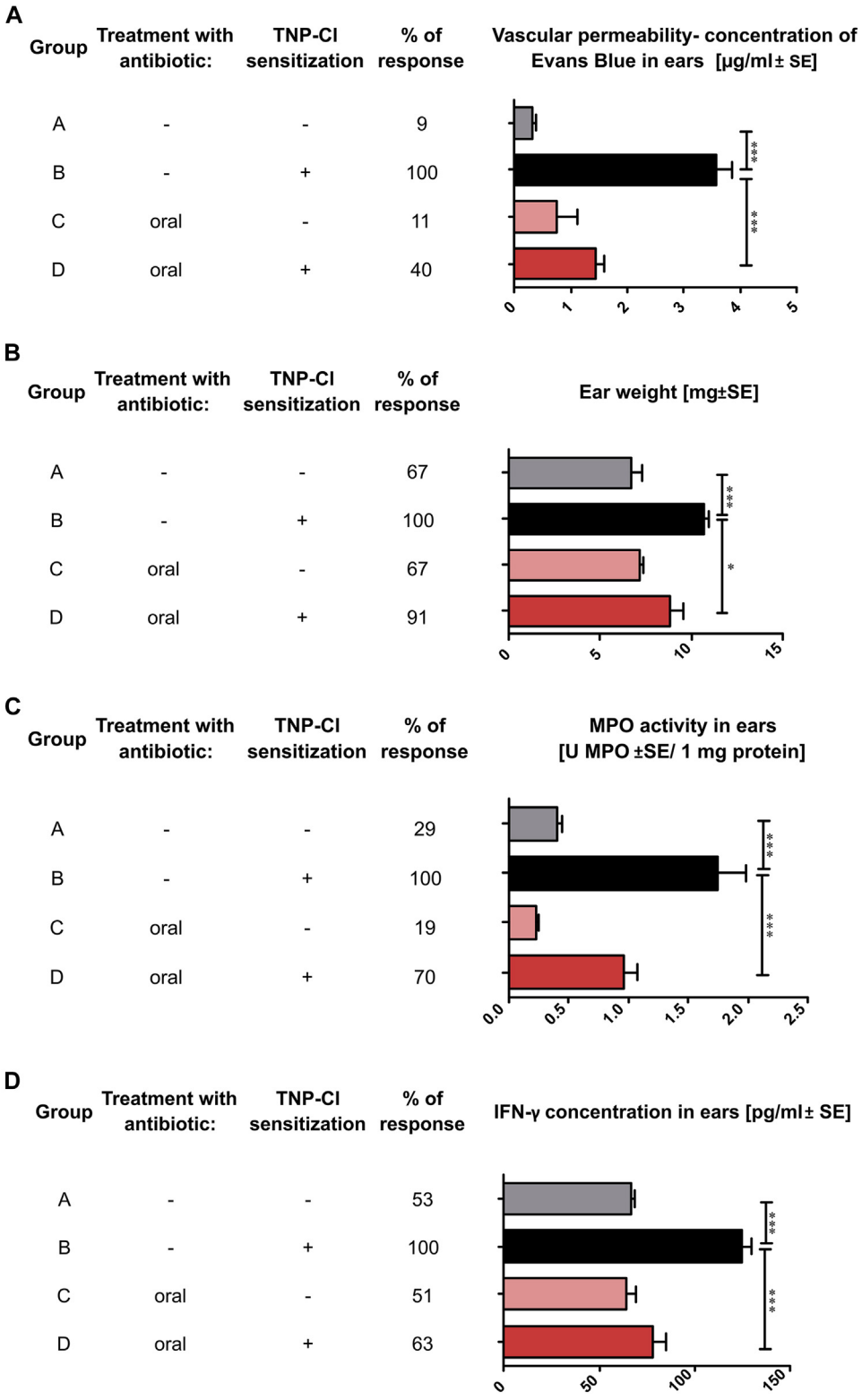


FIG 2. Oral treatment with antibiotic prior to CS induction inhibits inflammatory response in the ears. **A,** Vascular permeability. **B,** Ear weight. **C,** MPO activity in ear extracts. **D,** IFN-γ concentration in ear extracts. Results shown as means ± SEs. n = 10 to 14 (**A, B,** and **D**), n = 12 **(C)**. **P* < .05 and ****P* < .001. *U*, Unit.

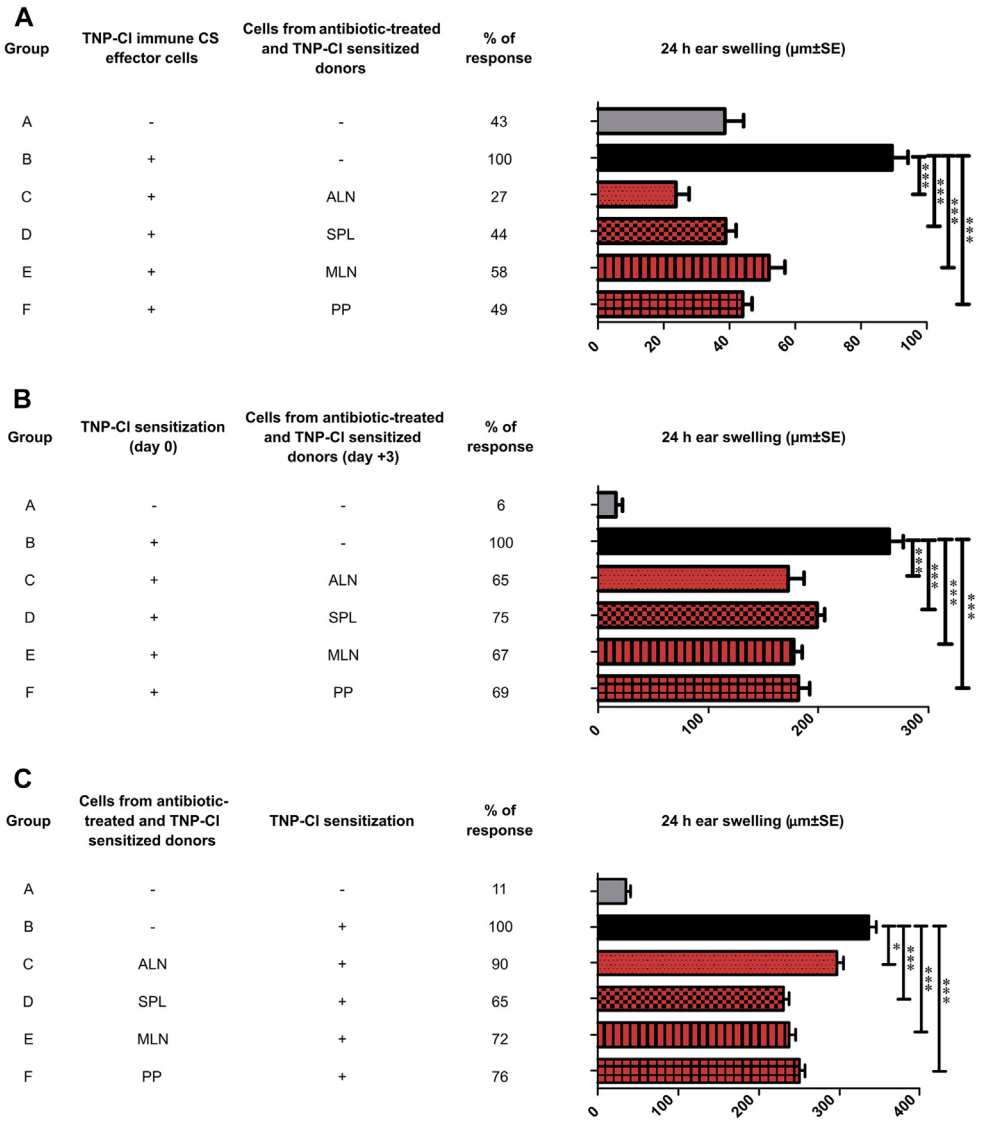


FIG 3. Oral treatment with antibiotic induces cells that suppress CS *in vivo*. Transfer of ALNCs or SPLCs or MLNCs or PP cells from donors treated orally with enrofloxacin and then sensitized. **A,** "Transfer out." **B,** "Transfer in" prior to allergen challenge. **C,** "Transfer in" prior to immunization. Results shown as means ± SEs. n = 10 to 14 (**A** and **B**), n = 8 to 12 **(C)**. **P* < .05 and ****P* < .001.

mice on the day of TNP-Cl contact immunization (day 0). These transferred cells interfere with the induction phase of the CS response (Fig 3, *C*, Groups C-F vs B).

Oral antibiotic treatment prior to TNP-Cl sensitization induces distinct populations of regulatory cells. As shown above, pretreatment with antibiotic prior to hapten sensitization results in the induction of regulatory cells in ALN, SPL, MLN, and PP. To determine which type of the regulatory cells is involved in the suppression of CS in our model, we phenotyped ALN, SPL, MLN, and PP cells by flow cytometry after staining with different fluorochrome-conjugated antibodies. Gating strategy is provided in Fig E1 in this article's Online Repository at www.jacionline.org. We found that the percentage of both TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ regulatory T (Treg) and $CD19^+B220^+CD5^+IL\text{-}10^+$ regulatory B (Breg) cells in PP were increased in mice orally treated with antibiotic followed by TNP-Cl sensitization when compared with hapten-sensitized mice that had not been treated with antibiotic (Fig 4, *A*, Group D vs B). Moreover, we observed nonsignificant increase of TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg cells in PP in mice treated with antibiotic without hapten sensitization, compared with the antibiotic-untreated mice (Fig 4, *A*, Group C vs A). Fluorescence-activated cell sorting (FACS) analysis of MLN showed that antibiotic treatment and hapten sensitization increased the frequency of both TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg and TCR $\alpha\beta^+CD4^+IL\text{-}10^+$ Tr1 cells when compared with antibiotic-untreated, hapten-sensitized mice (Fig 4, *B*, Group D vs B). Interestingly, we also found an increased frequency of TCR $\gamma\delta^+$ $IL\text{-}10^+$ lymphocytes in ALN and SPL of antibiotic-treated, hapten-sensitized mice compared with the animals with hapten sensitization in the absence of antibiotic treatment (Fig 4, *C* and *D*, Group D vs B).

Adoptive transfer of purified regulatory cell populations inhibits CS *in vivo*. To test the suppressive function of the regulatory cells induced by antibiotic treatment, we purified

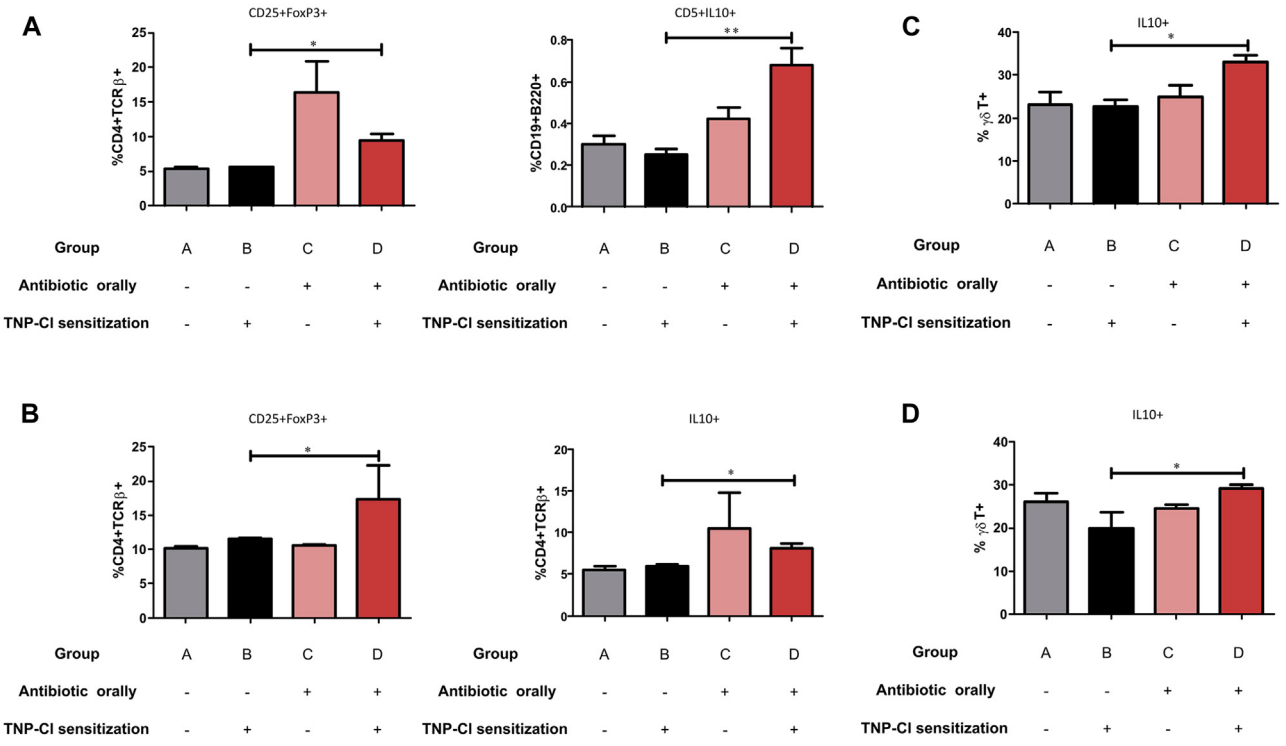


FIG 4. Oral treatment with antibiotic prior to TNP-Cl sensitization induces various populations of regulatory cells. Water (Groups A and B) or water with enrofloxacin (Groups C and D) receiving mice were (Groups B and D) or were not (Groups A and C) sensitized with TNP-Cl prior to FACS analysis of lymphoid organs. **A,** Percentage of TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg and $CD19^+B220^+CD5^+IL\text{-}10^+$ Breg cells in PP. **B,** Percentage of TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg and TCR $\alpha\beta^+CD4^+IL\text{-}10^+$ Tr1 cells in MLN. **C,** Percentage of TCR $\gamma\delta^+IL\text{-}10^+$ lymphocytes in ALN. **D,** Percentage of TCR $\gamma\delta^+IL\text{-}10^+$ lymphocytes in SPL. Results shown as means ± SEs. n = 4 to 5. $*P < .05$ and $**P < .01$.

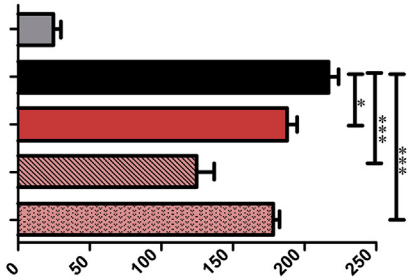
$CD4^+CD25^+$ cells or $CD19^+$ cells from PP of antibiotic-treated and hapten-sensitized donors and then transferred $CD4^+CD25^+$ (3.9×10^5), $CD19^+$ (3.3×10^5), or whole PP cells (1×10^7) into naive mice prior to TNP-Cl immunization. Each of these populations suppressed CS, although the suppression by $CD4^+CD25^+$ cells was greater (Fig 5, *A*, Groups D and E vs B). Our results suggest that Treg and Breg cells may be involved in the observed immunoregulation. We also tested the regulatory cells from MLN using the same approach. Total MLNCs (1×10^7) and purified $CD4^+CD25^+$ cells (6×10^5) from MLNs of antibiotic-treated and hapten-sensitized mice equally inhibited CS reactions in the recipients (Fig 5, *B*, Group D vs B). Finally, we tested the immune regulatory function of the TCR γδ cells that were observed to be increased after antibiotic treatment. We found that transfer of 5×10^7 unpurified ALNs or SPLCs of antibiotic-treated and hapten-sensitized donors downregulated CS response in the recipient mice (Fig 5, *C* and *D*, Group C vs B). Similar to the unpurified ALNs and SPLCs, purified TCR γδ from ALNs (1.5×10^6) or SPLCs (1×10^6) of antibiotic-treated and hapten-sensitized donors downregulated CS response in the recipient mice (Fig 5, *C* and *D*, Group D vs B).

Treatment with antibiotic shifts the bacterial population toward an anti-inflammatory profile. Oral antibiotic treatment significantly reduced both aerobic and anaerobic bacteria number in the gut. Recent studies indicate that some gut bacteria can promote anti-inflammatory responses, whereas others can induce inflammatory reactions.[25] To test whether the observed downregulated CS response in antibiotic-treated mice is the result of dysbiosis, we evaluated the composition of some common gut microbiota using RT-PCR. We found that some species of gut bacteria changed significantly between treated and untreated mice, whereas other bacterial species remained unaffected (Fig 6). Among the tested species, the relative abundance of *Enterococcus* spp and *Lactobacillus* were similar between untreated and antibiotic-treated mice (Fig 6, *A*, Group B vs A), whereas antibiotic treatment significantly increased the relative abundance of *Clostridium coccoides* (cluster XIVa) and *C coccoides–Eubacterium rectale* (cluster XIVab) compared with untreated mice (Fig 6, *B*, Group B vs A). Antibiotic treatment also increased the relative abundance of *Bacteroidetes* and *Bifidobacterium* spp compared with untreated mice (Fig 6, *C*, Group B vs A). In contrast, antibiotic treatment significantly decreased the relative abundance of segmented filamentous bacteria (SFB) compared with the control mice (Fig 6, *D*, Group B vs A).

Direct transfer of gut flora modulates CS response *in vivo*. To determine the direct role of gut microbiota in downregulation of CS response associated to antibiotic treatment, we took 2 approaches to foster microbiota exchange between mice. First we performed a cohousing experiment by keeping mice that were previously antibiotic-treated or untreated for 2 weeks in the same housing, followed by sensitization with TNP-Cl and testing for CS immune response. We found that naive mice cohoused with antibiotic-treated animals show decreased CS

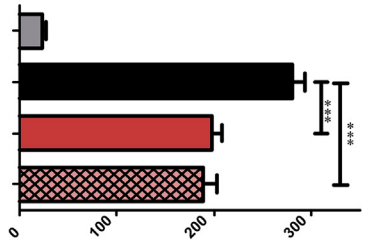
A

Group	Transfer of regulatory cells	TNP-CI sensitization	% of response
A	-	-	11
B	-	+	100
C	whole PP	+	87
D	CD4+CD25+	+	62
E	CD19+	+	82



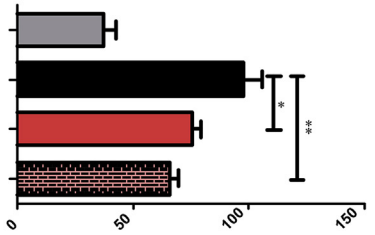
B

Group	Transfer of regulatory cells	TNP-CI sensitization	% of response
A	-	-	8
B	-	+	100
C	whole MLN	+	70
D	CD4+CD25+	+	67



C

Group	Transfer of regulatory cells	TNP-CI sensitization	% of response
A	-	-	37
B	-	+	100
C	whole ALN	+	73
D	TCRγδ+	+	67



D

Group	Transfer of regulatory cells	TNP-CI sensitization	% of response
A	-	-	8
B	-	+	100
C	whole SPL	+	52
D	TCRγδ+	+	68

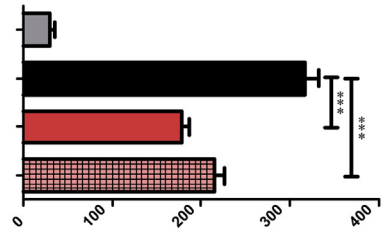


FIG 5. Transfer of purified regulatory cells prior to TNP-CI immunization inhibits CS *in vivo*. Whole immune cells from PP, MLN, ALN, or SPL or adequate magnetic cell sorting-purified cells from each organ were transferred to naive recipients prior to hapten immunization and CS test. **A,** Transfer of purified $CD4^+$ $CD25^+$ or $CD19^+$ cells from PP. **B,** Transfer of purified $CD4^+$ $CD25^+$ cells from MLN. **C,** Transfer of purified TCR γδ from ALN. **D,** Transfer of purified TCR γδ from SPL. Results shown as means ± SEs. n = 8. $*P < .05$, $**P < .01$, and $***P < .001$.

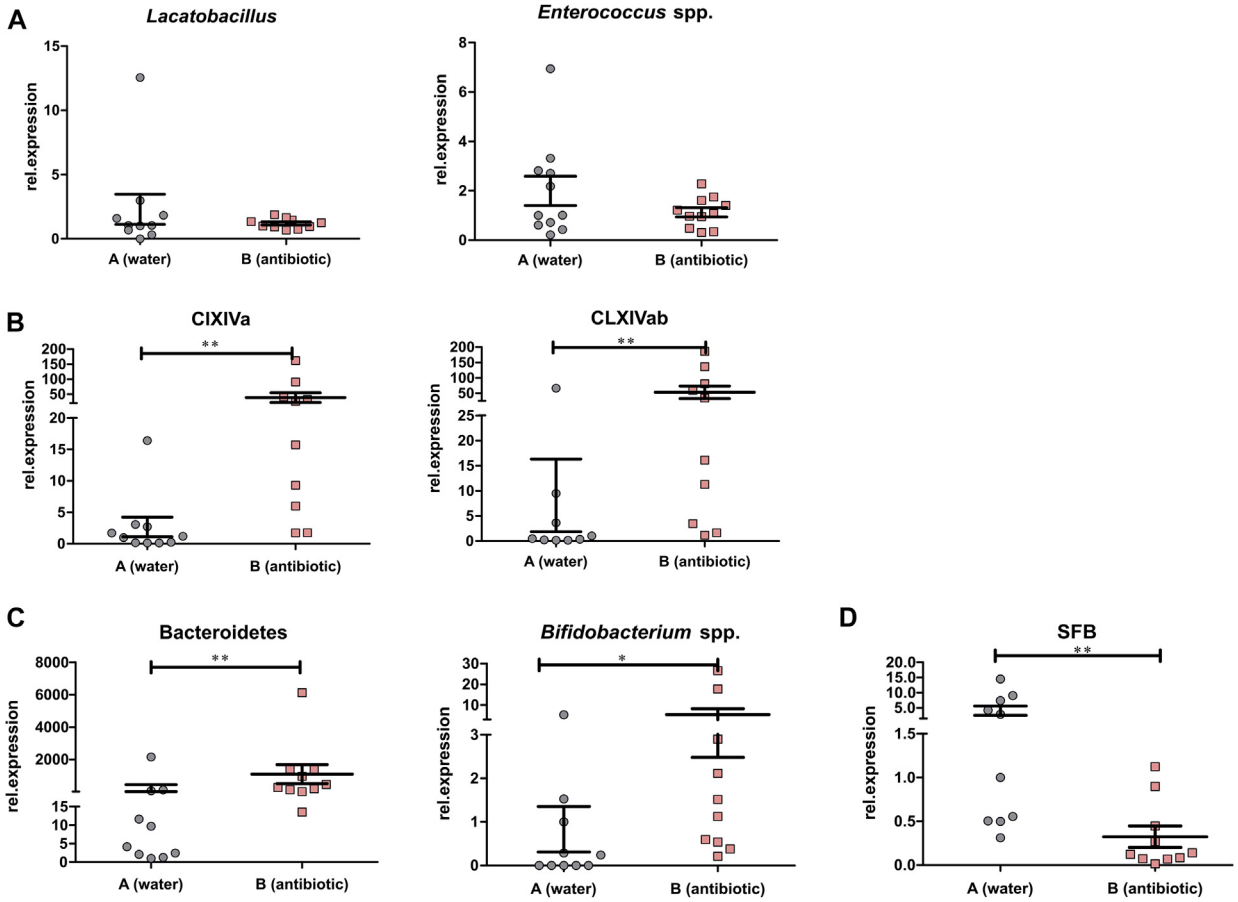


FIG 6. Treatment with antibiotic shifts the bacteria profile toward anti-inflammatory flora in mice treated with water containing enrofloxacin. Relative abundance of bacterial-conservative 16S rDNA fragments in gut: **A,** *Lactobacillus* and *Enterococcus* spp; **B,** *C coccoides* (cluster XIVa) and *C coccoides–E rectale* (cluster XIVab); **C,** *Bacteroidetes* and *Bifidobacterium* spp; **D,** SFB. Results shown as means ± SEs. n = 11. **P* < .05 and ***P* < .01.

compared with control mice (Fig 7, *A*, Group E vs B). The suppression of CS response in this group was similar to the suppression seen in the antibiotic-treated mice with or without cohousing (Fig 7, *A*, Group E vs C, D, and F). Next, we transferred gut microbiota from the mice with or without antibiotic treatment directly to naive mice through oral gavage, followed by induction of the CS response. The mice that received gut bacteria from antibiotic-treated donors showed decreased CS after TNP-Cl sensitization (Fig 7, *B*, Group C vs B), whereas there was no effect on CS response when the naive recipients were given gut microbes from non-antibiotic-treated donors (Fig 7, *B*, Group D vs B). Furthermore, the suppressed CS response seen in antibiotic-treated mice was reversed by oral transfer of gut microbes from non-antibiotic-treated mice (Fig 7, *C*, Group E vs C and D), whereas gavage of naive mice with gut flora from non-antibiotic-treated control animals did not affect CS (Fig 7, *C*, Group F vs B).

DISCUSSION

There is mounting evidence that the homeostasis of the gut microbiome is important for the balance and regulation of the immune system in both animals and humans. Recent studies have shown that microbiota play a crucial role in fostering the development of numerous T-cell subsets including T_H17 and Treg lymphocytes.[26] Germ-free mice have imbalanced T_H1/T_H17 responses that are beneficial in some autoimmune disorders, including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis,[27,28] but that appear to be detrimental for others, such as type 1 diabetes. The involvement of microbiota in the development of autoimmunity and other immune-mediated diseases has also been revealed by observing the effect of removing some gut bacteria with antibiotics.[13,14] It has been shown that adult mice treated with broad spectrum antibiotics develop ameliorated forms of animal models of T_H17-mediated psoriasis[29] and T_H1-mediated EAE[13] but show no effects in T_H2-mediated allergic responses.[30] On the other hand, broad spectrum antibiotics administrated early in life increase susceptibility to T_H17-mediated psoriasis[29] and to T_H2-mediated AD.[30] Increasing evidence suggests that imbalance in homeostasis of gut bacterial species is associated with atopic disorders.[12,31,32] Metagenome sequence analyses of the gut microbiome from patients with AD showed that enrichment of a subspecies of the major species of gut bacteria *Faecalibacterium prausnitzii* is strongly associated with AD.[33] It has been revealed that operational taxonomic units belonging to *F prausnitzii*

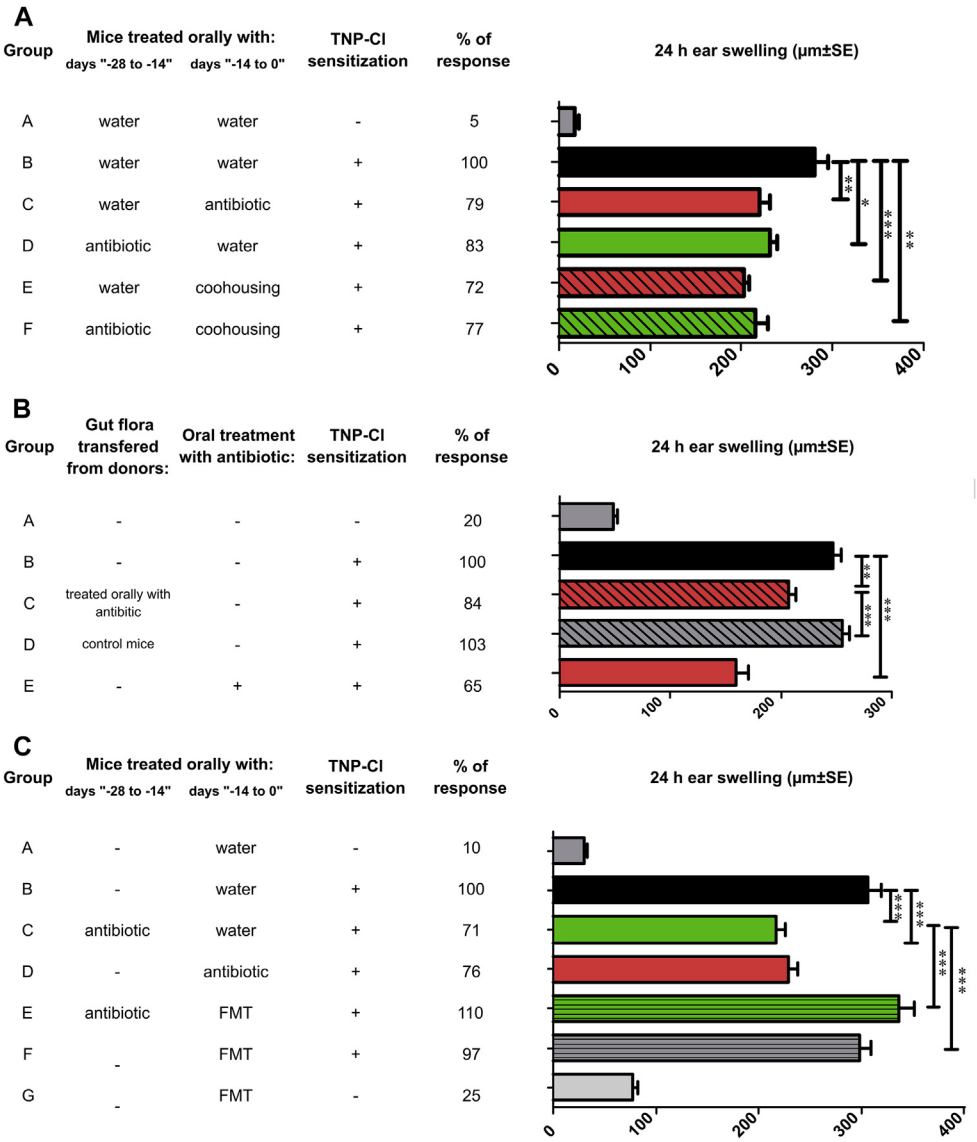


FIG 7. Transfer of gut flora modulates CS response *in vivo.* **A,** Cohousing experiment. **B,** Transfer of gut flora from enrofloxacin-treated donor mice. **C,** Transfer of gut flora from naive mice. Results shown as means ± SEs. n = 10 to 12 **(A)**, n = 10 to 14 **(B)**, n = 8 **(C)**. **P* < .05, ***P* < .01, and ****P* < .001. *FMT,* Fecal microbiota transplantation.

F06 are significantly more abundant in the AD-associated microbiota. Song et al[33] postulate that interactions between dysbiosis in *F prausnitzii* and dysregulation of gut epithelial inflammation might underlie the chronic progression of AD.

The influence of microbiota on CS response is not well explored. A few studies suggest that the proliferation of *Bifidobacterium pseudolongum* in the intestinal tract could be partially responsible for the reduction of 2,4-dinitro-1-fluorobenzene–induced CD8 Tc1-mediated CS response in mice,[34,35] and oral administration of the probiotic *Lactobacillus casei* alleviates CD8 Tc1-mediated CS to 2,4-dinitro-1-fluorobenzene.[36] Currently there are no studies, however, investigating the role of microbiota in $CD4^+$ T_H1-mediated CS responses. The novel findings of our study are, first, that the same antibiotic, enrofloxacin, administrated via different routes leads to different effects on CS *in vivo*. Second, we show that oral administration of enrofloxacin disturbs the natural composition of gut microbiota and induces different regulatory immune cells that suppress CS inflammatory response. Third, we show that the suppression of CS inflammatory response can be transferred to a new host by each population of regulatory cells. Fourth, we show that cohousing the mice with altered gut microbiota or fecal matter transfer can confer the protection to the new hosts. Finally, we show that the anti-inflammatory effect mediated by altered gut microbiota can be reversed by introducing normal gut microbiota. It is interesting that systemic administration of the same antibiotic, through *i.p.* injection, did not affect CS response *in vivo*, suggesting the importance of antibiotic activity within the intestinal lumen. Our study provides strong evidence that gut microbiota play an important role in skin allergic

inflammatory response. Concordantly, we find decreased CS responses after treatment with enrofloxacin prior to TNP-Cl sensitization in mice with highly divergent MHC haplotypes, namely non-obese-diabetic and BALB/c mice, suggesting that the observed phenomenon does not depend on the MHC background (data not shown).

The treatment with the antibiotic either orally or *i.p.* did not affect animal body weight, suggesting that the observed modulation of the CS response is not a result of side effects related to antibiotic treatment. The culture of gut content showed that oral but not *i.p.* administration of the antibiotic significantly reduced the number of both aerobic and anaerobic gut bacteria. Our findings are in line with recent data showing that reduction of gut commensal bacteria after oral, but not *i.p.,* treatment with antibiotic impairs the development of EAE.[13] Analyzing the composition of gut microbiota, we find an increase of *Bifidobacterium* spp, in agreement with the study by other investigators showing that consumption of prebiotic fructo-oligosaccharide, which is associated with proliferation of *B pseudolongum* in the intestinal tract of mice, reduces CS.[37]

The CS seen after transfer of ALNC and SPLC from antibiotic-treated and TNP-Cl-sensitized mice is weaker compared with CS after transfer of ALNC and SPLC isolated from non-antibiotic-treated, TNP-Cl-sensitized mice. However, this experiment did not answer the question of whether antibiotic treatment prior to hapten sensitization affects the induction of CS-effector cells themselves or is involved in the induction of regulatory cells. To answer this question, 2 approaches have been taken in our study. Using a "transfer out" protocol, we show that cotransfer of CS-effector cells with immune cells (ALNC or SPLC or MLNC or PP cells) isolated from antibiotic-treated donors results in decreased CS response when compared with the control mice that received CS-effector cells alone. This strongly suggests that immune cells from antibiotic-treated mice contain regulatory cells. Our results further demonstrate that the regulatory cells can act at both the induction and the effector phases of the CS response. Phenotyping the immune cells revealed an increased percentage of TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg cells in both MLN and PP of antibiotic-treated mice compared with nontreated mice. This is also in line with a study by Ochoa-Repáraz et al[13] that shows an increase of $Foxp3^+$ Treg cells in MLN and cervical LN of antibiotic-treated mice. In addition to $Foxp3^+$ Treg cells, we observe an increase of TCR $\alpha\beta^+CD4^+IL\text{-}10^+$ lymphocytes in MLN of antibiotic-treated mice compared with nontreated mice. Our results suggest that alteration of gut microbiota can induce both TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg cells in MLN and $IL\text{-}10^+$ Tr1 cells.[38] Interestingly, phenotypic analysis in our study also reveals an increase of Breg cells that are $CD19^+B220^+CD5^+IL\text{-}10^+$ in PP of antibiotic-treated mice. A study in an EAE model system has shown that antibiotic-treated mice have an increased percentage of $CD19^+B220^+$, IL-10-producing, $CD5^+$ Breg cells in peripheral lymph organs.[39] It has been reported that $CD19^+$ B cells also can negatively regulate CS responses.[40] Our results further indicate that IL-10-producing TCR $\gamma\delta^+$ cells are among the immune regulatory cells from antibiotic-treated mice. Our findings add another population of microbiota-associated regulatory cells that has not been appreciated to date. We have reported previously the involvement of TCR γδ cells in negative regulation of CS response.[41] Our current study indicates that oral administration of antibiotic prior to hapten sensitization induces 4 different subsets of regulatory cells. In this study, we show the ability of these 4 populations to suppress the CS response *in vivo* by transferring each purified cell subset individually. These experiments prove that each cell population indeed has the ability to suppress the CS response *in vivo* after adoptive transfer. Our study also provides evidence that alteration of gut microbiota due to the oral administration of antibiotics may affect the development of regulatory cells.

It is known that antibiotic treatment decreases not only the number of bacteria but also the diversity of the microbiota. This effect can last from days to weeks after the cessation of antibiotic administration, and it is possible that some bacterial species could be depleted from the community permanently.[42] Moreover, antibiotics can affect the immune system, favorably or harmfully, through the change of bacterial composition. Recent studies have shown that *Bacteroides fragilis* increases the suppressive activity of Treg cells, whereas certain *Clostridium* strains increase the frequency of IL-10-producing Tregs.[43,44] Other studies reported that gavage of commensal bacteria of the *Lactobacillus* or *Bifidobacterium* genera can induce Treg cells.[45,46] However, the studies of SFB in mice have demonstrated that SFB is closely associated with the induction of T_H17 lymphocytes.[47] In this study, we demonstrate that oral treatment with the antibiotic increases the relative abundance of *C coccoides* (cluster XIVa), *C coccoides–E rectale* (cluster XIVab), *Bacteroidetes,* and *Bifidobacterium* spp, all of which have been shown to promote anti-inflammatory responses.[25] In contrast, oral antibiotic treatment decreases the relative abundance of SFB, which are known to promote IL-17-mediated inflammatory response.[47] Our results suggest that antibiotic-induced dysbiosis of gut microbiota favors an anti-inflammatory status and ameliorates contact sensitivity of skin inflammatory response. This is further confirmed by cohousing naive mice with antibiotic-treated mice and by gut microbiota transfer from antibiotic-treated mice to naive mice, both of which suppress CS responses in naive mice. More importantly, we show that the CS response can be restored through oral transfer of control gut bacteria to antibiotic-treated animals, suggesting that changes in the gut flora can revert to a natural homeostatic state through oral transfer. It is noteworthy that the route of antibiotic administration is essential for our findings presented in this study, as systemic antibiotic administration via the *i.p.* route had little effect on the immune regulation of CS.

In summary, our work shows that oral treatment with the broad spectrum antibiotic enrofloxacin modifies gut microbiota composition to promote an anti-inflammatory response as seen in reduced CS responses. The observed changes in microbiota promote the induction of different populations of regulatory cells that inhibit CS *in vivo*. The suppression of the CS response induced by antibiotic effects on the microbiota is reversible, as gavage of antibiotic-treated mice with control gut flora restores CS reactions. Thus, targeted manipulation of gut microbiota, leading to increased level of anti-inflammatory bacteria could be a therapeutic strategy to ameliorate allergic contact dermatitis.

We would like to thank Dr Philip W. Askenase for his help.

Key messages

- **Oral treatment with broad spectrum antibiotic enrofloxacin leads to gut dysbiosis associated with increased levels of anti-inflammatory bacteria.**
- **An alteration in gut microbiota profile favors induction of a variety of regulatory cells that inhibit CS in mice.**
- **Transfer of antibiotic-modified gut microbiota inhibits CS, indicating that targeted modulation of gut microbiota could have therapeutic potential.**

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METHODS

Sensitization and elicitation of CS *in vivo*

Mice were sensitized by topical application of 0.15 mL of 5% TNP-Cl in acetone-ethanol mixture (1:3) to the shaved abdomen and chest. Control mice were shaved and painted with the acetone-ethanol mixture alone as a sham sensitization. Four days later, animals were challenged on both sides of the ears with 10 μL of 0.4% TNP-Cl in olive oil–acetone mixture (1:1). Resulting ear thickness was measured prior to testing with a micrometer (Mitutoyo, Tokyo, Japan) by an observer unaware of the experimental groups and then again at 24 hours after allergen challenge. Negative control consisted of littermate sham-sensitized animals that were similarly challenged. The ear swelling was expressed in μm ± SE.[E1] Ear swelling was further confirmed by the measurements of ear weight, vascular permeability, MPO activity, and IFN-γ concentration in ear extracts. Additionally, concentrations of anti-TNP IgG1 and IgG2a were measured in mouse sera by ELISA. The immunomodulatory influence of microbiota on the severity of CS was evaluated by detection of gut bacteria composition using PCR. The primers sequences used to detect selected gut bacteria species are provided in Table E1.

Vascular permeability test

To assess very early changes in vascular permeability, TNP-Cl immunized or naive mice were challenged with 10 μL of 0.4% TNP-Cl and injected with 1% Evans blue dye (83 μg/g body weight) 23 hours later. One hour after Evans blue injection, mice were anesthetized and sacrificed. Ears were removed, and a 6-mm diameter punch from each ear was made with biopsy punch (cat# BP60; Fray Products Corp, Buffalo, NY). Ear punches were transferred to tubes containing 1 mL of formamide. After 18 hours' incubation at 37°C, the samples were centrifuged and the optical density of Evans blue in the supernatant was read at 565 nm against a blank containing formamide.[E1]

MPO assay

Neutrophil infiltration to the inflamed ears was indirectly quantitated using a MPO assay, as described previously.[E1] Ears were removed 24 hours post allergen challenge, and a 6-mm diameter punch from each ear was made. Biopsies were collected from the distal site of CS responses and were homogenized in 0.5% hexadecyltrimethylammonium bromide pH = 6.0 (50 mg of tissue/mL). The homogenates were freeze-thawed 3 times, centrifuged at 40,000*g*. Then 0.1-mL aliquots were mixed with 2.9 mL of phosphate buffer (pH = 6.0) containing 0.167 mg/mL o-dianisidine dihydrochloride and 5×10^{-4}% H_2O_2 and incubated at 25°C for 20 minutes. The absorbance was measured at 460 nm in 96-well flat bottom plates. MPO activity was expressed in units per protein concentration (U/mg of protein).

In vitro measurement of IFN-γ in CS ear extracts

To determine local production of IFN-γ in elicited TNP-Cl CS, mice were immunized with 5% TNP-Cl or sham-sensitized and allergen challenged with 10 μL of 0.4% TNP-Cl 4 days later. Ears were removed 24 hours post allergen challenge, and a 6-mm diameter punch from each ear was made. Biopsies were collected from the distal site of CS ear responses. The biopsies were frozen rapidly in liquid N_2 and were subsequently thawed and extracted in 300 μL of cold PBS containing a mixture of proteinase inhibitors on ice with a tissue microhomogenizer.[E1] Concentration of IFN-γ was measured by ELISA with the use of BD OptEIA Set (BD Biosciences, San Diego, Calif).

FACS analysis

ALNs, SPLs, MLNs, and PPs were isolated and single-cell suspensions prepared and stained prior to analysis. Cell subpopulations were analyzed using fluorochrome-conjugated mAbs. For the analysis of T-cell subpopulations, CD4-PerCP-Cy5.5 and TCR β-APC-Cy7 (BioLegend, San Diego, Calif), CD25-APC, and TCR γδ-PE (BD Biosciences, San Jose, Calif) and CD4-FITC (Life Technologies Corporation, Carlsbad, Calif) were used. For B-cell identification, B220-PE-Cy7 and CD19-PerCP-Cy5.5 (eBioscience Inc, San Diego, Calif) and CD5-FITC (BD Biosciences) were used.

Treg cells were determined by staining with anti-FoxP3-PE mAb (BD Biosciences) using the mouse Treg staining kit (eBioscience) according to manufacturers' instructions. To detect intracellular IL-10, intracellular cytokines staining was performed. Single-cell suspension from lymphoid tissues were cultured for 4 hours with phorol 12-myristate 13-acetate, ionomycin (Sigma Chemical Co, St. Louis, Mo), and Golgi Plug (eBioscience), followed by surface-markers staining of T and B cells. After washing, the cells were fixed and permeabilized followed by staining with IL-10-PE-Cy7 or IL-10-PE. The cells were analyzed with a FACS Canto II (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed using FACSDiva software (Becton Dickinson). Gating strategies are provided in Fig E1.

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E1. Majewska-Szczepanik M, Strzępa A, Marcińska K, Wen L, Szczepanik M. Epicutaneous immunization with TNP-Ig and Zymosan induces TCR $\alpha\beta^+CD4^+$ contrasuppressor cells that reverse skin-induced suppression via IL-17A. Int Arch Allergy Immunol 2014;164:122-36.

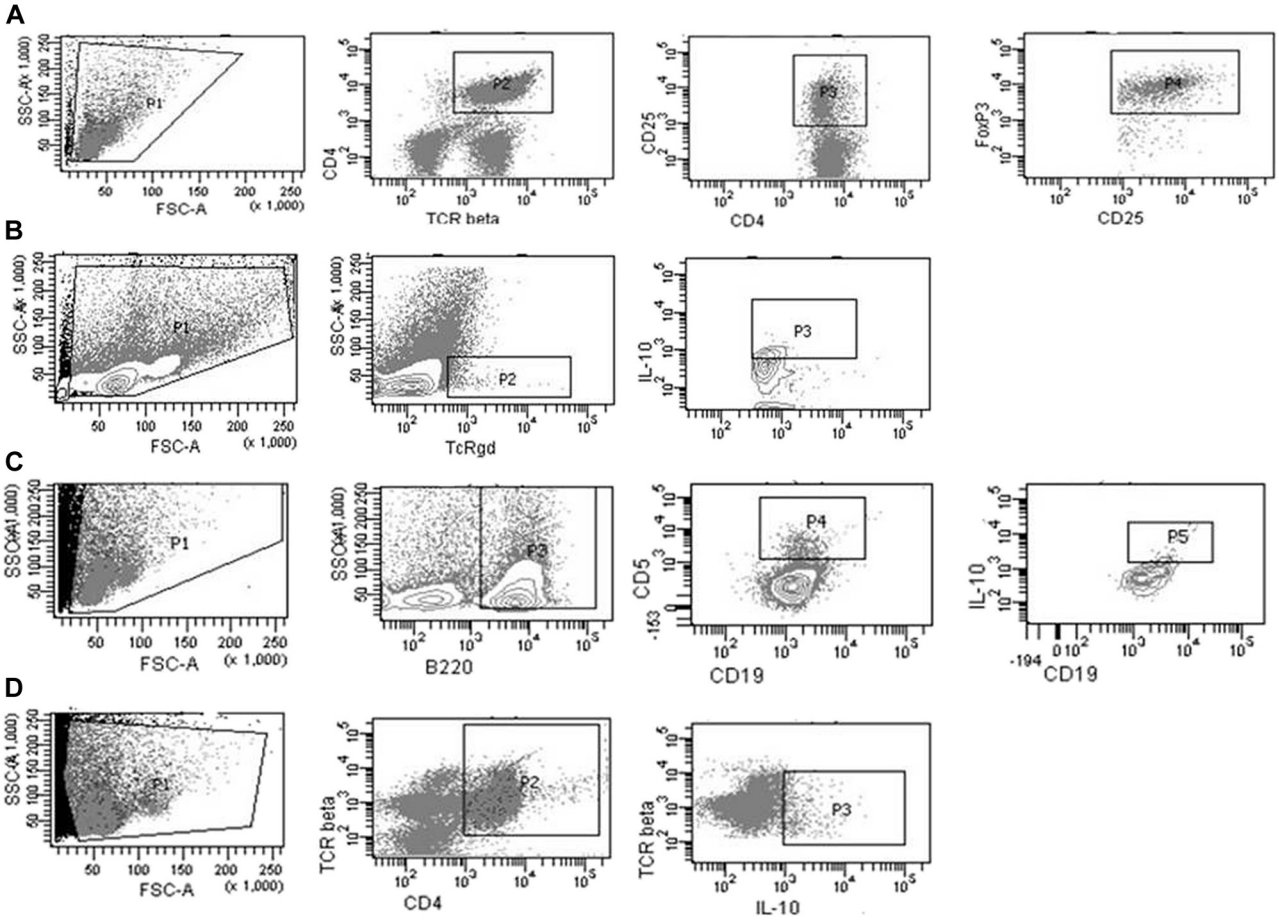

FIG E1. Representative flow cytometry gating strategies for: **A,** TCR $\alpha\beta^+CD4^+CD25^+FoxP3^+$ Treg; **B,** IL-10^+ TCR $\gamma\delta^+$ cells; **C,** $CD19^+B220^+CD5^+$IL-10^+ Breg; **D,** TCR $\alpha\beta^+CD4^+$IL-10^+ Tr1. *FSC-A*, Foward scatter; *SSC-A*, side scatter; *TcRgd*, TCR $\gamma\delta$.

TABLE E1. The primers sequences used to detect selected gut bacteria species

	Primers
C coccoides–E rectale subgroup (cluster XIVab)	F: 5′-AAATGACGGTACCTGACTAA-3′ R: 5′-CTTTGAGTTTCATTCTTGCGAA-3′
C coccoides (cluster XIVa)	F: 5′-AAATGACGGTACCTGACTAA-3′ R: 5′-CTTTGAGTTTCATTCTTGCGAA-3′
Enterococcus spp	F: 5′-CCCTTATTGTTAGTTGCCATCATT-3′ R: 5′-ACTCGTTGTACTTCCCATTGT-3′
Lactobacillus	F: 5′-AGCAGTAGGGAATCTTCCA-3′ R: 5′-CACCGCTACACATGGAG-3′
Bacteroidetes	F: 5′-GGTGTCGGCTTAAGTGCCAT-3′ R: 5′-CGGA(C/T)GTAAGGGCCGTGC-3′
Bifidobacterium spp	F: 5′-TCGCGTC(C/T)GGTGTGAAAG-3′ R: 5′-CCACATCCAGC(A/G)TCCAC-3′
SFB	F: 5′-GACGCTGAGGCATGAGAGCAT R: 5′-GACGGCACGGATTGTTATTCA
Bacteria	F: 5′-TCCTACGGGAGGCAGCAGT-3′ R: 5′- GGACTACCAGGGTATCTAATCCTGTT-3′ (6-FAM)-5′-CGTATTACCGCGGCTGCTGGCAC-3′-(TAMRA)

F, Forward primer; *FAM*, 6-carboxyfluorescein; *R*, reverse primer; *TAMRA*, tetramethylrhodamine.